



0 211.1-NDH



METHODS USEFUL IN ENDOTOXIN BASED
PROPHYLAXIS AND THERAPY

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Number EG88896053/US

Date of Deposit May 2, 1995

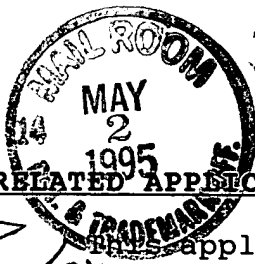
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RELATED APPLICATIONS

211.1-NDH

08432691



05/02/95

Insert B1/2
This application is a continuation-in-part of PCT Application PCT/US93/07453 filed August 9, 1993 which is a continuation-in-part of U.S. Patent Application Serial Number 07/928,930 filed August 12, 1992, now U.S. Patent No. *B1/2*

FIELD OF THE INVENTION

This invention relates to the treatment of endotoxin related endotoxemia. More particularly, it relates to the treatment of such poisoning via administration of various compositions which act to neutralize and/or ^{+D}remove endotoxins from the organism, as well as prophylaxis utilizing these compositions.

BACKGROUND AND PRIOR ART

Normal serum contains a number of lipoprotein particles which are characterized according to their density, namely, chylomicrons, VLDL^s, LDL^s and HDL^s. They are composed of free and esterified cholesterol, triglycerides, phospholipids, several other minor lipid components, and protein. Very low density lipoprotein (VLDL) transports energy, in the form of triglycerides, to the cells of the body for storage and use. As triglycerides are delivered, VLDL is converted to low density lipoprotein (LDL). Low density lipoprotein (LDL) transports cholesterol and other lipid soluble materials to the cells in the body, while high density lipoprotein (HDL) transports excess or unusable ^{Cholesterol} to the liver for elimination. Normally, these lipoproteins are in balance, ensuring proper

✓ delivery and removal of a lipid soluble materials. Abnormally low HDL can cause a number of diseased states as well as constitute a secondary complication in others.

Under normal conditions, a natural HDL is a solid particle with its surface covered by a phospholipid monolayer that encloses a hydrophobic core. Apolipoprotein A-I and A-II attach to the surface by interaction of the hydrophobic face of their alpha helical domains. In its nascent or newly secreted form the particle is disk-shaped and accepts free cholesterol into its bilayer. Cholesterol is esterified by the action of lecithin:cholesterol acyltransferase (LCAT) and is moved into the center of the disk. The movement of cholesterol ester to the center is the result of space and solubility limitations within the bilayer. The HDL particle "inflates" to a spheroidal particle as more and more cholesterol is esterified and moved to the center. Cholesterol ester and other water insoluble lipids which collect in the "inflated core" of the HDL are then cleared by the liver.

Anantharamaiah, in Segrest et al., Meth. Enzymol. 128: 627-647 (1986) describes a series of peptides which form "helical wheels", as a result of the interaction of the amino acids in the peptide with each other. Such helical wheels present a nonpolar face, and a polar face in their configuration. The reference shows, generally, that peptides can replace aproteins in these particles.

Jonas et al., Meth. Enzym. 128A: 553-582 (1986) have produced a wide variety of reconstituted particles resembling HDL. The

technique involves the isolation and delipidation of HDL by standard methods (Hatch et al., Adv. Lip. Res. 6: 1-68 (1968); Scanu et al., Anal. Biochem. 44: 576-588 (1971) to obtain apo-HDL proteins. The apoproteins are fractionated and reconstituted with phospholipid and with or without cholesterol using detergent dialysis.

Matz et al., J. Biol. Chem. 257(8): 4535-4540 (1982) describe a micelle of phosphatidylcholine, with apolipoprotein A1. Various ratios of the two components are described, and it is suggested that the described method can be used to make other micelles. It is suggested as well to use the micelles as an enzyme substrate, or as a model for the HDL molecule. This paper does not, however discuss application of the micelles to cholesterol removal, nor does it give any suggestions as to diagnostic or therapeutic use.

Williams et al., Biochem. & Biophys. Acta 875: 183-194 (1986) teach phospholipid liposomes introduced to plasma which pick up apoproteins and cholesterol. Liposomes are disclosed, which pick up apoprotein in vivo, as well as cholesterol, and it is suggested that the uptake of cholesterol is enhanced in phospholipid liposomes which have interacted with, and picked up apoproteins.

Williams et al., Persp. Biol. & Med. 27(3): 417-431 1984) discuss lecithin liposomes as removing cholesterol. The paper summarizes earlier work showing that liposomes which contain apoproteins remove cholesterol from cells in vitro more effectively than liposomes which do not contain it. They do not discuss in vivo use of apoprotein containing liposomes or micelles, and

counsel caution in any in vivo work with liposomes.

It is important to note that there is a clear and significant difference between the particles of the present invention, and the liposomes and micelles described in the prior art. The latter involve a bilayer structure of lipid-containing molecules, surrounding an internal aqueous core space. The structure of liposomes precludes filling the internal space with a lipid soluble component, however, and any molecular uptake of lipid soluble components is limited to the space defined between the two lipid layers. As a result, there is much less volume available for pick up and discharge of materials such as cholesterol and other lipid soluble materials than there is for the particles of this invention, which expand in a fashion similar to a balloon, with interior space filling with the material of choice.

Endotoxic shock is a condition, often fatal, provoked by the release of lipopolysaccharide (LPS) from the outer membrane of most gram negative ^{bacteria} ~~bacterial~~ (e.g., Escherichia coli; Salmonella typhimurium). The structure of the bacterial LPS has been fairly well elucidated, and a unique molecule, referred to as lipid A, which is linked to acyl chains via lipid A molecule's glucosamine backbone ^{is a component of LPS.} See Raetz, Ann. Rev. Biochem. 59: 129-170 (1990) in this regard.

The lipid A molecule serves as membrane anchor of a lipopolysaccharide structure ("LPS") and it is the LPS which is implicated in the development of endotoxic shock. It should be pointed out that LPS molecules are characterized by a lipid A type

structure and a polysaccharide portion. This latter moiety may vary in molecular details in different LPS molecules, but it will retain the general structural motifs characteristic of endotoxins. It would be incorrect to say that the LPS molecule is the same from bacteria to bacteria (see Raetz, supra). It is common in the art to refer to the various LPS molecules as "endotoxins", and this term will be used hereafter to refer to LPS molecules collectively.

In U.S. Patent No. 5,128,318 the disclosure of which is incorporated by reference, it was taught that reconstituted particles containing both an HDL associated apolipoprotein and a lipid capable of binding an endotoxin to inactivate it could be used as effective materials for alleviating endotoxin caused toxicity.

In the parent and grandparent applications cited in the Related Application section and incorporated by reference herein, it was disclosed that various other materials may be used to treat endotoxin caused toxicity. Specifically, it was found that apolipoproteins are not required in reconstituted particles, and that the reconstituted particle may contain a peptide and a lipid wherein the peptide is not an apolipoprotein.

It was also found by the inventors that endotoxin caused toxicity may be treated via sequential administration of either an apolipoprotein or a peptide followed by a lipid. Following sequential administration, the components assemble as a reconstituted particle and then act to remove endotoxin.

It was also found that at least some individuals possess native levels of apolipoprotein which are higher than normal levels such that effective endotoxemia therapy may be effectuated by administering reconstituted particles containing no apolipoprotein or peptide, but containing the lipid of the disclosure.

In addition, the invention disclosed in these applications involved the use of the reconstituted particles and the components discussed herein for prophylaxis against endotoxin caused toxicity, by administering prophylactically effective amounts to subjects in need of prophylaxis. Such subjects include patients suffering from infections or recovering from surgery. These patients sometimes have very low plasma HDL levels, sometimes as little as 20% of normal levels. It is highly desirable, in these cases, for early prophylaxis with HDL, so as to compensate for these drops.

It has now been found, quite surprisingly, that phospholipids may be used alone, or in combination with neutral lipids, as effective agents to alleviate and/or prevent endotoxemia. It is especially preferred to use phosphatidylcholines ("PC" hereafter), either alone, or in combination with other phospholipids, such as sphingolipids, in compositions which are essentially free of peptides and proteins, such as apolipoproteins or peptides derived

therefrom. Neutral lipids such as mono-, di-, and triglycerides may be combined with the phospholipids, as long as the total amount of neutral lipids is below certain weight percents when the compositions are used in the form of an intravenous bolus. When
5 used in other forms of administration, such as intravenously for example, by continuous infusion, the weight percents are not so critical, but are desirable.

Particularly preferred embodiments of the invention are those compositions where the neutral lipid is cholesterol ester, or a
10 mixture of cholesterol ester and triglycerides.

The invention is described in greater detail in the disclosure which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows how reconstituted particles containing Apo-A1,
15 phospholipid and cholate form.

Figure 2 shows the reception of LPS molecules by reconstituted particles.

Figure 3 shows experiments in which a peptide in accordance with the invention as used to study reduction of endotoxin caused
20 toxicity in a mouse model.

Figure 4, labelled prior art, shows the formation of helical wheels by various peptides.. See Anantharamaiah, supra.

Figures 5A and 5B show results obtained when various compositions

were tested in a model which determined the neutralization of endotoxin via determining TNF release in a human whole blood model. Figure 5A shows the role of protein, and 5B that of phospholipid. The compositions tested included natural lipoproteins (VLDL, LDL, HDL), reconstituted HDL ("R-HDL"), and INTRALIPID® compositions, as well as emulsions containing phospholipid and protein.

Figures 6A and 6B compare the role of triglyceride (a neutral lipid), and phosphatidylcholine, a phospholipid, in the same model.

Figure 7 presents reported information on toxicity associated with administration of various PC and PC/TG compositions in a mouse model, using a 70% toxicity model where E. coli LPS is administered.

Figure 8 shows data comparable to that secured for the human whole blood assay, supra, but using phospholipid with unesterified cholesterol, sphingomyelin, or mixtures of both, in place of triglycerides.

Figures 9A and 9B show results comparable to those shown in figures 5A and 5B, except that in these new figures, phospholipid, unesterified cholesterol and/or sphingomyelin are mixed with triglycerides or esterified cholesterol as the neutral lipid.

Figure 10 compares results obtained from cholesterol ester and triglyceride containing emulsions, in the in vivo mouse model.

Figure 11 graphs the theoretical amounts of triglycerides released

into the blood following administration of various TG containing compositions, with toxicity thresholds. "TPN" stands for "total parenteral nutrition" while "RI" stands for compositions in accordance with the invention.

5 DETAILED DESCRIPTION OF THE EMBODIMENTS

Example 1

Studies were carried out to determine the survival rate of mice challenged with S. typhimurium endotoxin. Outbred male, ✓ Swiss-Webster mice received either saline ^{Solution} (20 mice), reconstituted HDL particles (40 mice), or reconstituted peptide 18A (20 mice), via injection through the tail vein. The particulars of the injection materials are as follows:

a. HDL particles

15 Particles were prepared from apo-Hu-HDL (85%-AI; 15% AII and apo C), reconstituted with 95% pure egg phosphatidylcholine (2:1 W/W), using detergent dialysis, in accordance with Matz et al., J. Biol. Chem. 257: 4535-4540 (1982), and U.S. Patent No. 5,128,318, the disclosure of which is incorporated by reference.

b. peptide particles

20 The peptide 18A has the amino acids sequence:

Asp-Trp-Leu-Lys-Ala-Phe-Tyr-Asp-Lys-Val-Ala-Gly-Lys-Leu-Lys-Glu-Ala-Phe (SEQ ID NO: 1)

○ Samples of peptide were also mixed and reconstituted with 95% pure eggs phosphatidylcholine as per Matz et al., supra (2:1 w/w),

and U.S. Patent No. 5,128,318 also using detergent dialysis. The resulting particles are identical to those disclosed in U.S. Patent No. 5,128,318 except that a peptide component was present, rather than the apo-HDL of the Matz and patent references.

5 Within fifteen minutes of administration of the reconstituted material, the mice were administered, intraperitoneally, 10 mg/kg body weight of Salmonella LPS. The criterion for evaluation was survival. Figure 3 presents these results, and indicates nearly 4 fold superiority over the saline control. The synthetic peptide
10 is almost as effective as the reconstituted apo-HDL containing particles.

Example 2

Factors which affect the LPS-mediated stimulation of $\text{TNF-}\alpha$ while preserving the integrity of interaction between plasma
15 proteins, and cellular elements of blood, can be appropriately studied in an in vitro, human whole blood system. Such a system was used to determine which of the components of lipoproteins is important in neutralizing LPS.

Materials tested were reconstituted high density lipoprotein
20 \checkmark (R-HDL), natural plasma lipoproteins (VLDL, ^{LDL}~~LDL~~, HDL), lipoprotein deficient serum (LPDS), and the triglyceride rich emulsion 20% INTRALIPID® (a mixture of triglycerides and phospholipids).

All particles described herein were made via the same protocol, which involved mixing a phospholipid, sphingomyelin or
25 phosphatidylcholine, triolein, and/or unesterified cholesterol

ester, dissolved in chloroform, and weighing it into a flask. Vitamin E (0.02% w/v) was added as antioxidant. A dry lipid film was then prepared by blowing nitrogen or argon gas over the sample. A volume of non pyrogenic saline was then added to the flask, followed by mixing on a vortex mixer until all lipid was suspended. The solution was then homogenized in a high pressure homogenizer. Samples containing phosphatidylcholine (PC), with or without triolein, were cycled through the homogenizer 10 times at 20,000 psi. Samples containing cholesterol ester with one or more other lipids were cycled through 15-20 times at 30,000 psi. Homogenized solutions were filtered through 0.45 μ m syringe filters, and the filtrate was stored at room temperature until used (within three days).

Blood was collected in a heparinized tube, diluted with Hank's Balanced Salt Solution ("HBSS" hereafter), or the material to be tested, dissolved in HBSS. The resulting material was transferred to Starstedt tubes (250 ul/tube). LPS was dissolved in pyrogen free saline containing 10 mM HEPES, and added (2.5 ul) to a final concentration of 10 ng/ml. After incubation for four hours at 37°C, tubes were chilled to 4°C, followed by centrifugation at 10,000xg for 5 minutes. Supernatant was collected, and assayed for determination of TNF- α , using a commercially available ELISA.

Table 1, which follows, compares the compositions of the materials tested. Figures 5A and 5B present the results. Data are plotted as amount of TNF- α produced, plotted against concentration of added protein (figure 5A), and phospholipid (figure 5B).

Logarithmic scales were used, in order to display the wide range of concentrations used, with 10^0 equal to 1 mg/ml. All whole blood incubations contained 10 ng/ml of E. coli 0111:B4 LPS, supplemented with one of the compositions, as the key for figures 5A and 5B show.

The fact that the materials differ in effectiveness when protein content is plotted (figure 5A), while being very similar when phospholipid content is plotted (figure 5B) suggest that the phospholipid is the important component. This is confirmed by the finding that a protein free lipid emulsion is more effective than is natural HDL, but less effective than R-HDL. Protein does not appear important to the neutralization.

7147 Composition of natural lipoproteins and reconstituted HDL

Lipoprotein		TC	TG	PC	Protein
Class	Density (g/ml)	Weight %			
VLDL	<1.006	22	53	18	7
LDL	1.007-1.063	48	11	22	20.9
HDL	1.063-1.21	18	8	22	52
R-HDL	1.063-1.21	-	-	79	21
LPDS	>1.21	0	0	2	98
Intralipid	-	1	93	6	0

20 Example 3

As the next step, protein free lipid emulsions, containing different amounts of neutral lipid, were tested in human whole blood. The same in vitro human whole blood assay as set forth in example 2 was used. Figures 6A and 6B present these results. In these studies, LPS-dependent, TNF- α production is plotted against concentration of added triglyceride (figure 6A), or phospholipid (figure 6B). The compositions, as indicated by the key, contained (by weight) 7% triglyceride ("TG"), 45% TG, 89% TG, 94% TG, R-HDL, or phospholipid without TG, (shown in figure 6B only). An 89% TG composition is a 10% INTRALIPID® formulation, while 94% TG refers to 20% INTRALIPID. In all other tests, egg phosphatidylcholine (PC), and triolein were used.

These results show that the protein free compositions, when

compared via triglyceride content, are very different. They are very similar when tested via phospholipid (PC) content. This confirms the role of phospholipid, especially since phospholipid alone is effective, but less so than emulsions containing up to 45% TG.

Example 4

The work then proceeded to in vivo experiments in a mouse model, which is accepted as a reliable system for predicting human efficacy.

In these experiments, mice were injected, in bolus form, with sufficient amounts of the formulations described in example 3 as well as others (pure phosphatidylcholine, 7% TG, 45% TG, 71% TG, 80% TG, 89% TG, 94% TG), or a saline control, to provide doses of phospholipid (either 200 mg/kg or 400 mg/kg), together with 25 mg/kg of E. coli 0111:B4 LPS. The control group received intravenous physiological saline in a volume sufficient to match the volume of emulsion. Survival after 72 hours is presented in figure 7.

Overall survival in the control group was $29\% \pm 8\%$ (mean: 63 animals in 8 experiments). Each preparation was tested in a minimum of 3 experiments on 18 or more animals.

PC alone had a modest protective effect, not statistically significant at the 95% confidence level, while 7%, 45% and 71% TG compositions significantly improved survival. The 80% and 89% TG compositions were marginally effective, while the 94% TG decreased

survival.

When the dose was increased to provide 400 mg/kg of PC both the 89% and 94% TG emulsions significantly decreased survival time, probably due to TG poisoning, as explained infra.

5 Example 5

The work described in examples 2-4 established that phospholipids are an active agent useful in inhibiting endotoxemia. The fact that non-polar lipids other than triglycerides may form emulsions with phospholipids other than PC suggested that others
10 ✓ may be tried. Exemplary are ^{sphingomyelin}~~spingomyelin~~ (another phospholipid), and unesterified cholesterol (a polar neutral lipid), and mixtures of these. So, too, esterified cholesterol (a nonpolar ester), squalene (a hydrocarbon), and vitamin E (a nonpolar antioxidant) may be used. A series of experiments were designed to test these,
15 using the human whole blood assay of example 2, supra, and the mouse survival assay of example 4.

Emulsions were prepared, in the manner described supra, using pure phosphatidylcholine, phosphatidylcholine with 10% (wt/wt) unesterified cholesterol, 10% (wt/wt) sphingomyelin, or 10% total
20 of a mix of both. Emulsions were added to whole blood, at a concentration of 100 mg/dl, with reference to PC, and 10 ng/ml of LPS. The mixture was incubated, and TNF- α release measured.

The results are shown in Figure 8. TNF- α production was substantially reduced with PC alone. Emulsions containing
25 unesterified cholesterol, sphingomyelin, or the mix of both, were

also suppressive of TNF- α release.

Example 6

The whole blood assay was also used to determine the effect of unesterified cholesterol and/or sphingomyelin to neutral lipid containing emulsions. Again, the emulsions were added at 100 mg/dl PC. The various compositions (wt/wt) are set forth in the following table.

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Emulsion	Composition
PC with 45% TG	55:45
PC + TG + C	54.4:45.3:0.3
PC + TG + SP	51.6:43.0:5.4
PC + TG + C + SP	51.4:42.9:0.3:5.4
PC + CE	54.5:45.5
PC + CE + C	54.4:45.3:0.3
PC + CE + SP	51.6:43.0:5.4
PC + CE + C + SP	51.5:42.9:0.3:5.4

Figures 9A and 9B display the results. PC emulsions made with either neutral lipid, with or without additional polar lipids, demonstrated inhibition. Again, the LPS concentration used is a 70% lethal dose. The cholesterol ester containing emulsions are less effective than are TG containing emulsions, while those emulsions containing unesterified cholesterol did not suppress TNF- α as well as those emulsions which did not contain it. Adding sphingomyelin to the emulsions appeared to improve suppression of

TNF- α production.

Example 7

✓ Cholesterol ester containing emulsions were tested in an in
✓ vivo model (i.e., that used in example 4), with a lethal dose of
5 endotoxin. Emulsions were prepared with PC and TG, or PC and
cholesterol ester (CE), and were administered to provide a single
✓ bolus dose of 200 mg/kg of PC, together with 25 mg/kg of E. coli
0111:B4 LPS (a lethal dose), through the tail vein. Control groups
received intravenous physiological saline in a volume to match the
10 volume of emulsion.

In figure 10, the data compare the results from the CE and TG
containing emulsions. Each emulsion was tested in a minimum of two
experiments, using a total of 16 or more animals.

15 As shown, emulsions containing 7% or 45% CE (wt%)
significantly improved survival. These results, taken with those
of example 6, show that CE can be substituted for TG to create
emulsions that neutralize endotoxin.

Example 8

Protein-free emulsions of phospholipid with triglyceride effectively block TNF- α production in whole blood stimulated with LPS. In theory, these emulsions might also be effective in vivo if they can be administered safely in doses that provide protective concentrations of phospholipid in plasma. Our previous experiments with R-HDL suggest that the minimum ^{effective} dose of phospholipid is approximately 200 mg/kg. Using this dose and a plasma volume of 4.5% of body weight, one can calculate the concentration of triglyceride expected in plasma following administration of a series of emulsions with increasing triglyceride content. The result is shown in figure 11 as a smooth line curving upward with increasing weight percent TG. Plasma TG concentrations rarely rise above 1000 mg/dl in healthy adults even after a fatty meal. Pancreatitis is reported in patients with plasma TG above 2000 mg/dl (Farmer, et al., Amer. J. Med. 54: 161-164 (1973); Krauss, et al., Amer. J. Med. 62: 144-149 (1977); Glueck, et al., J. Lab. Clin. Med. 123: 59-61). Plasma TG above 4000 mg/dl is extremely rare and cause for serious concern. The last two thresholds are shown by horizontal lines in the figure above. Administration of either 10% or 20% INTRALIPID® in a dose to provide 200 mg/kg phospholipid is expected to raise plasma TG concentrations (see the two open circles) well above the safe limits. By contrast, administration of emulsions containing 7%, 45%, 71% or 78% (solid squares left to right) raises plasma TG to 136, 477, 1300 or 2000 mg/dl respectively. Emulsions with TG content up to ~50% are

expected to be free of toxicity from TG.

The foregoing examples detail the invention which involves, in one aspect, the alleviation or prevention of endotoxemia in a subject via administering an effective amount of a phospholipid with which an endotoxin associates. The association of phospholipid and endotoxin is then removed from the subject via standard biological processes well known to anyone familiar with processes via which lipoprotein particles are removed. Association of the endotoxin with the phospholipid inactivates it.

The subject being treated is preferably a human, but the practice of the invention is equally applicable in a veterinary context as well.

"Alleviation" as used herein refers to treatment to ease the burden of endotoxemia caused by any of the various endotoxins produced by, e.g., gram negative bacteria (S. typhimurium, E. coli, etc.). Prophylaxis may be accomplished by administering the agent at a point where the subject is in or about to be in, a situation where endotoxin exposure may result. Classically, this occurs during surgery. Thus, a subject who is about to experience a surgical procedure may have the active ingredient administered preparatory to the procedure.

The effective amount of phospholipid necessary for treatment of the subject can vary. In general, a dose up to about 200 mg of phospholipid per kilogram of body weight of the subject is preferred, although the amount may drop, or increase, depending upon the severity of the endotoxemia or the degree of risk in the

context of the prophylaxis.

It is desirable to administer the phospholipids in compositions which also contain neutral lipids, but this is not necessary, as neutral lipid free emulsions of phospholipids are also envisioned. The desirability of the administration with phospholipids results from the fact that the neutral lipids and phospholipids associate into particles which resemble the lipoproteins, but differ therefrom in that they contain no protein of peptide components, which are of course, always present in the lipoproteins.

Especially desirable forms of treatment are those where the phospholipid is a phosphatidylcholine, such as egg yolk phosphatidylcholine, soy based phosphatidylcholine or a sphingolipid. With respect to the neutral lipids, it is preferred to use cholesterol ester or triglyceride, but other neutral lipids, such as squalene or other hydrocarbon oils, di- and mono-glycerides and antioxidants such as vitamin E may also be used.

The form in which the compositions may be administered can vary, with a bolus or other intravenous forms being especially preferred. When a bolus form is used, and the composition contains triglyceride, e.g., some care must be given in dosing. It is fairly well known that triglycerides are toxic if administered in too large an amount. The artisan of ordinary skill, however, can easily formulate the compositions so that the risk of triglyceride poisoning is reduced, or eliminated. In general, when a bolus form is used, the compositions should contain no more than about 80

weight percent ~~by weight~~ of triglyceride or other neutral lipid, preferably no more than 70 weight percent. Most preferably, the compositions should contain no more than about 50 weight percent, of neutral lipid, when a bolus is administered.

5 When non-bolus forms are employed, however, such as other intravenous forms, the risk of poisoning is decreased. Nonetheless, the ranges delineated supra are preferred for intravenous, or other forms of administration, although it must be understood that they are not required. Preferably, a dose of up to about 200 mg per kg of body weight of phospholipid is administered. Administration of up to about or even up to about 800 mg/kg is also feasible. Doses are general, however, and will vary depending upon the subject and the form of administration.

As indicated, supra, the protein and peptide free formulations
 15 ~~require that at least one phospholipid be present; and preferably~~
~~at least one neutral lipid be present; optionally, these~~
 additional materials such as sterols (e.g., cholesterol, β -sitosterol), esterified or unesterified lipids (e.g., cholesterol ester or unesterified cholesterol), hydrocarbon oils such as
 20 ~~squalene, antioxidants such as vitamin E, but these are not~~
 required. Of course, more than one phospholipid, and/or more than one neutral lipid may be used in any formulation.

Other aspects of the invention will be clear to the skilled artisan, and need not be repeated here.

25 It will be understood that the specification and examples are illustrative but not limitative of the present invention and that

other embodiments within the spirit and scope of the invention will suggest themselves to those skilled in the art.

T2410X

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Levine, Daniel M.; Parker, Thomas S.;
Rubin, Albert L; Gordon, Bruce R; Saal, Stuart D.
- (ii) TITLE OF INVENTION: Methods Useful in Endotoxin Based
Prophylaxis and Therapy
- (iii) NUMBER OF SEQUENCES: 9
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- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette, 5.25 inch, 360 kb
storage
 - (B) COMPUTER: IBM PS/2
 - (C) OPERATING SYSTEM: PC-DOS
 - (D) SOFTWARE: Wordperfect
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: PCT/US93/07453
 - (B) FILING DATE: 9-AUGUST-1993
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 07/928,930
 - (B) FILING DATE: 12-AUGUST-1992
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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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 Glu Ala Phe

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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 5 10 15
 Lys Ala Phe

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Asp Trp Leu Lys Ala Phe Tyr Asp Lys Ala Glu Lys Leu Lys Glu
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 Ala Phe

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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Pro Lys Leu Glu Glu Leu Lys Glu Lys Leu Lys Glu Leu Leu Glu
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Lys Leu Lys Glu Lys Leu Ala
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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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Val Ser Ser Leu Lys Glu Tyr Trp Ser Ser Leu Lys Glu Ser Phe
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(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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Lys Glu Ser Leu Ser
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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```

Val Ser Ser Leu Leu Ser Ser Leu Leu Ser Ser Leu Lys Glu Tyr
           5                               10               15
Trp Ser Ser Leu Lys Glu Ser Glu Ser
           20

```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Pro Val Leu Asp Glu Phe Arg Glu Lys Leu Asn Glu Glu Leu Glu
 5 10 15
 Ala Leu Lys Gln Lys Met Lys
 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Pro Leu Ala Glu Asp Leu Gln Thr Lys Leu Asn Glu Asn Val Glu
 5 10 15
 Asp Leu Arg Lys Gln Leu Val
 20